FLUOROMETRIC ASSAY TO DETECT THE GERMINATION OF *BACILLUS ANTHRACIS* SPORES AND THE GERMINATION INHIBITORY EFFECTS OF ANTIBODIES

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ABSTRACT

A sensitive assay for germination of spores of the anthrax agent was developed using a fluorescence reader. Significant inhibition of germination was detected for spores initially treated with antispore or antitoxin antibodies. Antigens specific to ungerminated spores that stimulate potentially protective antibodies are being identified and their role in inhibition of germination and in immune protection against anthrax will be determined. The germination of spores of *Bacillus anthracis* is typically detected by alterations in spore refractility, stainability, and absorbance (OD560). We have developed a more quantitative, semi-automated procedure for detecting germination by using a microtiter kinetic reader for fluorescence spectrophotometry (Biotek FL600). The procedure was based on the increase in fluorescence of spores with time during their incubation in germination medium containing Syto-9, a fluorescent nucleic acid-binding dye which stained germinated *B. anthracis* but not dormant spores. A colorless, defined medium containing equal parts of L-alanine, adenosine, and casamino acids provided low levels of background fluorescence, stimulated germination, and yielded reasonable germination kinetics. Antibodies (Abs) to the protective antigen (PA) component of the anthrax toxins of *B. anthracis* have been shown to inhibit *in vitro* germination. Regression analyses of the germination data obtained with the reader yielded parameters (*i.e.*, the difference between the baseline and maximal fluorescence values [a coefficient] and the area under the curve) that gave significant differences between spores pretreated with anti-PA or antispore antibodies and buffer or nonimmune sera. We are testing sera from vaccinated or passively-immunized animals to determine the correlation between serum germination inhibitory activity, titers of serum anti-PA or antispore antibodies, and survival after lethal challenge.

INTRODUCTION

The recent occurrence of 22 cases of bioterrorism-associated anthrax has confirmed the known potential of *Bacillus anthracis* to be used as a biological weapon. Early detection and aggressive intervention with antibiotics resulted in the survival of 6 of the 11 patients with the usually lethal inhalational form of anthrax[1]. However, infected and/or exposed individuals treated with antibiotics are at risk of developing inhalational anthrax when the therapy is terminated and dormant spores are able to germinate[2]. The protection of individuals exposed to the spores might be enhanced by preventing germination of the infectious spores into the replicating bacilli. The bacilli produce the anthrax edema and lethal toxins, composed of the cell-

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binding protective antigen (PA) in association with the edema factor (EF) or the lethal factor (LF), respectively[3]. Antibodies (Abs) to PA have an essential role in immune protection[4]. In addition to their toxin-neutralizing activity, anti-PA Abs have anti-spore activities. These include the stimulation of phagocytosis of spores by macrophages and the inhibition of spore germination *in vitro*[5]. The significance of these activities *in vivo* is unknown. However, the recent findings that: 1) toxin-associated proteins may be expressed early in the infectious process[7]; 2) anti-PA Abs bind to the spore surface and decrease the level of spore germination[6]; and 3) formaldehyde-inactivated spores can serve as a protective vaccine against anthrax challenge in guinea pigs[8] led to the hypothesis that antibodies to spore-surface-expressed antigens can block spore germination or render spores more susceptible to phagocytosis and ultimately killing by host cells.

The treatment of spores of the Ames strain of *B. anthracis* with anti-PA Abs inhibits their germination upon incubation of the treated spores in complex or defined germination media[6]. Germination can be detected microscopically, *i.e*., as a loss in refractility or increase in stainability (Fig. 1), as well as by a decrease with time in absorbance at A_{560}[6].

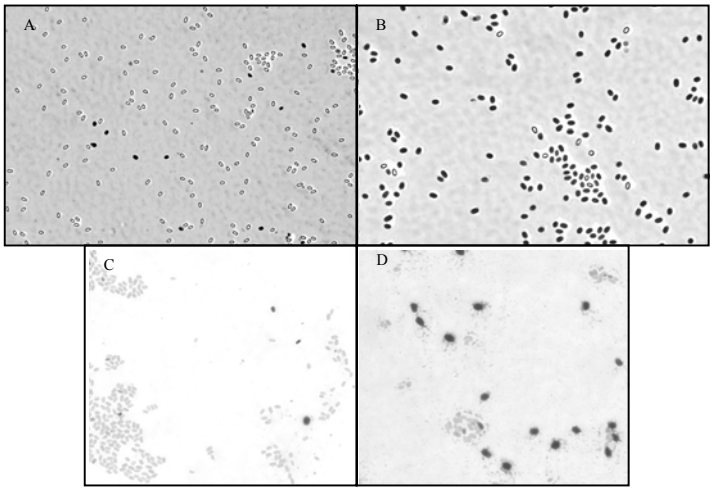

Figure 1.The extent of germination of dormant spores *in vitro* can be detected microscopically as a decrease in refractility under phase contrast and an increase in staining by dyes.

Phase micrographs of spore suspensions containing mainly ungerminated, refractile spores (A) or germinated, nonrefractile spores (B). Malachite green spore stains of suspensions with mostly ungerminated green-stained spores and rare germinated ones stained purple with the Wright/Giemsa counterstain (C), or approximately equal numbers of ungerminated and germinated organisms (D).

To further study the Ab-associated effects, we developed a quantitative and semi-automated assay for germination using a microtiter kinetic reader for fluorescence spectrophotometry (Biotek FL600). The latter detected germination based on the increase in fluorescence of spores with time during their incubation in a defined germination medium with a fluorescent nucleic acid-binding dye that stained germinated but not ungerminated spores of *B. anthracis*.

MATERIALS AND METHODS

STRAINS AND SPORE PREPARATIONS

Strains of *B. anthracis* used included the virulent encapsulated toxigenic Ames and Vollum 1B strains and the non-encapsulated toxigenic Sterne vaccine strain. Spores were prepared and purified from broth cultures of the strains, as described[6,9,10], and were used only if ≥95% were refractile (ungerminated) as determined by phase microscopy. The spores were activated by heating at 65 °C for 30 min just prior to use in assays[9].

SERA AND ANTIBODIES

Immune sera from mice, guinea pigs, rabbits, and monkeys were obtained from animals vaccinated with AVA, purified PA or PA-producing VEE viral replicon particles[13]. Rabbit antisera designated anti-Sterne PA and anti-rPA antisera, were from animals hyperimmune to PA purified from the native Sterne strain or ΔSterne-1 (pPA102) CR4, respectively[6,11,12]. Affinity-purified rabbit anti-PA IgG (1.05 mg IgG/ml) was obtained by chromatography of rPA antisera over a PA antigen column followed by a Protein A column. IgG from sera collected from non-immune normal rabbit (2.05 mg IgG/ml, NRS) was prepared similarly, as described previously[6]. Affinity-purified polyclonal rabbit anti-spore antibodies, provided by R. Bull (NNMC), were prepared against a spore immunogen consisting of a pool with equal numbers of inactivated spores of four strains, Ames, Vollum , New Hampshire, and Sterne. Prechallenge sera were obtained from nonhuman primates immunized with AVA (as described in Table 1 below), and from guinea pigs immunized twice intramuscularly with recombinant PA or native PA purified from the Sterne strain and then challenged im with ≥100 LD_{50} doses of Ames spores. The PA-replicon vaccine contains a nonreplicating derivative of the Venezuelan Equine Encephalitis RNA virus that expresses a cloned PA gene, as described previously[13,14].

MICROTITER FLUORESCENCE ASSAY FOR GERMINATION

The spores were incubated on ice for 30 min with IgG or serum samples in the wells of a sterile polypropylene round bottom microtiter plate. The tray was centrifuged to pellet the spores (3000 rpm, 15 min), the supernatants are removed, and the pellets resuspended in 50 µl water. Suspensions were transferred to wells of a black clear bottom 96-well tray containing 50 µl of 2x-concentrated germinant and Syto-9 dye (Molecular Probes), a fluorescent stain for nucleic acids that stains germinated but not ungerminated spores. The germinant used was a modification of one described previously[15], and consisted of a solution of L-alanine (1.67 mg/ml), adenosine (1.67 mg/ml) and 0.33% casamino acids and was stored at -20C. An aliquot was thawed and diluted 1/8 in water ("2x") just prior to use. A two-component germinant consisting of 0.25 mM L-alanine and 1 mM inosine was also used (data not shown). The tray was read in the Biotek FL600 reader with filters for excitation (485)/emission (530). Sample fluorescence was recorded automatically at 60 sec intervals for 30 to 60 min.

STATISTICAL ANALYSIS

The germination kinetics of spores treated with different sera or IgG preparations were analyzed by using a four-parameter logistic regression model available on a PC software program (SigmaPlot). Differences were found in two of the regression parameters and in the areas under the curve, as described further in the Results. Standard methods were used to determine statistical significance and to analyze the data and included the mean, standard error of the mean (SEM), analysis of variance, Z-tests and unpaired Student's t-tests. In comparing groups, a p

value of $\leq$0.05 was considered to indicate a significant difference. The association between the *in vitro* germination inhibitory activity of sera from guinea pigs immunized with PA and survival after challenge was analyzed by using Cox's proportional hazards model as described elsewhere[16].

RESULTS

DEVELOPMENT OF A FLUORESCENCE ASSAY FOR GERMINATION

An assay for the germination of spores of *B. anthracis* was developed using the Biotek FL600 microtiter fluorescence reader. The assay detected germination based on the increase in fluorescence of spores with time during their incubation in media found to stimulate germination with low levels of background fluorescence. Spore samples are added to wells of a 96-well plate containing germinant, the plate read on the FL600, and the data reported in Relative Fluorescence Units (RFUs), as shown in Fig. 2.

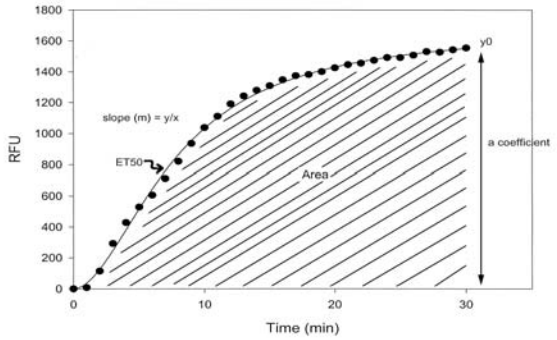

Figure 2. Germination assay using the Biotek FL600 microtiter fluorescence reader.

A regression line of the fluorescence kinetics for 30 one min readings of one spore sample is shown. A four-parameter logistics regression model fit the sigmoidal data and provided parameters that could be used to statistically compare the rates of germination of different spore samples, as shown on the graph, including the slope of the curve (m), the inflection point, when 50% of the maximal germination observed (ET_{50}), the maximum saturation point (asymptote) of the curve (y0), the difference between the baseline and the plateau (a coefficient), and the area under the curve.

Significant differences compared to controls in the germination kinetics of antispore antibody- or anti-PA-pretreated spores were found in two of the regression parameters, the a coefficient and the areas under the curve. Additional parameters found to be useful in distinguishing differences in the germination inhibitory activity (GIA) were the RFU values read immediately upon exposure to germinant and the final total % increase in RFU (after the 30- or 60-min total exposure).

EFFECTS OF ANTISPORE ANTIBODIES ON SPORE GERMINATION

Pretreatment of spores with sera or IgG against either whole ungerminated spores (Fig. 3) or PA (Fig. 4) inhibited spore germination. The GIA was manifested by differences in the Areas under the fluorescence curve and the a coefficient, as described in figure1.

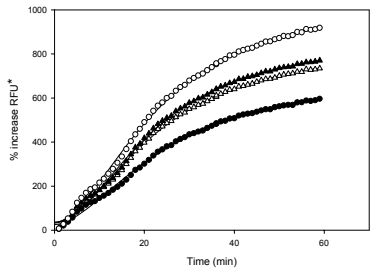

Figure 3.Incubation of spores of the Ames strain of *B. anthracis* with purified rabbit polyclonal Abs prepared against whole ungerminated spores.

Pretreatment of Ames spores with dilutions of the antispore Ab inhibited germination in the fluorescence assay in a dose-related manner. In the present assay, the germination inhibitory activity (GIA) was optimal at a 1/500 dilution (equivalent to 8.46 ug/ml). Dilutions of Ab: 1/500 = ●, 1/1000 = Δ, 1/2000 = ▲, and buffer alone = ○ *The RFU (% increase) value of a sample is the difference between the RFU at a given time in germination medium and the RFU at t0, expressed as a percentage of the latter.

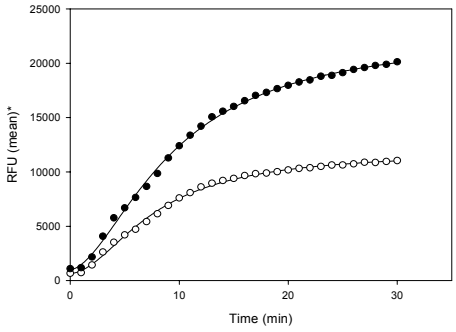

Figure 4. Inhibition of germination of spores of the Ames strain of *B. anthracis* by pretreatment with anti-PA Abs.

Ungerminated spores were pretreated with a 1/50 dilution of purified rabbit anti-rPA IgG (❍) or normal rabbit serum (NRS) IgG (●), and the spores then incubated in the alanine-adenosine-casamino acids germinant. The anti-PA-treated spores germinated to a significantly lesser extent than did the spores pretreated with normal rabbit IgG, as shown by the smaller area-under-the-curve and a coefficient values ($p<0.0001$ by Z-test). * The mean RFU value is the mean of duplicate values after subtraction of background (RFU of wells with no spores).

EFFECT OF *B. ANTHRACIS* STRAIN ON GERMINATION INHIBITION BY ANTI-PA

Anti-rPA Abs inhibited germination of strains Ames and Sterne spores but not of strain VollumIB (V1B) spores to a significantly greater extent that did NRS IgG (Fig. 5). The data suggest that V1B might express less of the anti-PA-reactive antigen on the spore surface than Ames and Sterne; this observation agrees with the absence of extractable PA from V1B spores as detected on SDS-PAGE[6].

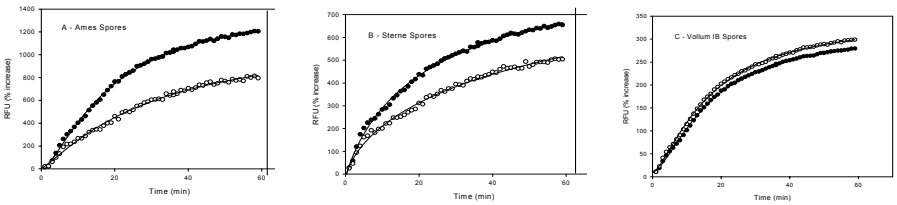

Figure 5.Ungerminated spores of the Ames, Sterne and V1B strains

These strains were prepared and purified under the same conditions and used at the same concentrations (5 – 8 x 106 spores/well). Germination of spores pretreated with anti-rPA IgG(❍) or NRS IgG (●), both diluted 1/100, is shown for spores of Ames (A), Sterne (B), and V1B (C). Anti-rPA Abs inhibited germination of Ames and Sterne spores [p = 0.017 (n=8 experiments) and 0.011 (n=2 experiments), respectively], but not of V1B spores (n=2 experiments), to a significantly greater extent that did NRS IgG.

SERUM GERMINATION INHIBITORY ACTIVITY: A POSSIBLE *IN VITRO* CORRELATE OF IMMUNITY

MICE

The association between protection, serum GIA, and serum anti-PA titer was examined in mice immunized with AVA or the PA-replicon vaccine[13]. Sera were collected from individual vaccinated mice prior to each vaccine dose and just prior to subcutaneous challenge with a lethal dose of *B. anthracis* strain Sterne.

PA-REPLICON-IMMUNIZED MICE

Spores were pretreated with either preimmune sera or sera from PA replicon-immunized C57Bl/6 mice prior to exposure to germinant. GIA was assayed by determining the ratio of the Area under the fluorescence curve of the prechallenge- compared to the preimmune-treated spores. The area ratio for the serum pair from immunized C57Bl/6J mice (n=10) correlated significantly with the anti-PA Elisa titers. (p = 0.0033, r = 0.855); ie., high anti-PA Ab titers in prechallenge sera were associated with strong germination inhibitory activity. However, there were insufficient numbers of nonsurvivors to determine the correlation between GIA or anti-PA titer and survival. In contrast to the C57Bl/6J mice, the inhibitory activity of PA replicon-immunized A/J mice (n= 16) was not significantly correlated with the anti-PA Elisa titer; and neither anti-PA titer nor GIA were significantly associated with survival. An example of the association between serum GIA

and anti-PA titer is illustrated in Fig.6. In this figure, the kinetics of germination and the results of regression analyses for spores treated with sequential sera from a C57Bl/6J mouse collected after each vaccine dose are shown.

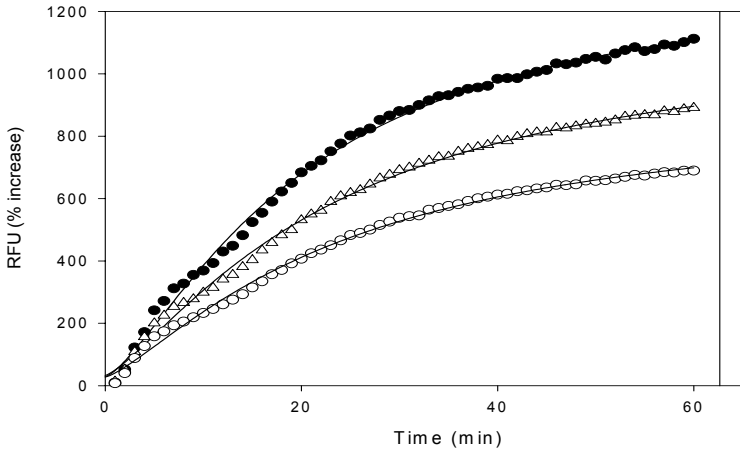

Figure 6. PA-replicon-immunized mice: Anti-PA titers and germination inhibitory activity of sequential sera from an immunized mouse. Serum #1 (preimmune): ●, serum #2: △, serum #5: ❍

Table 1. PA-replicon-immunized mice: Anti-PA titers and germination inhibitory activity of sequential sera from an immunized mouse.

Serum no.		Germination assay		Anti-PA	
Mouse	(No. vaccine doses)	a coefficient*	Area	antibody titer**	TTD (day)
C57Bl/6	#1 (preimmune)	1320	45,411	neg	
48-6	#2 (1 dose)	1116 (.0016)	36,159	25,600	
	#3 (2 doses)	nd	nd	819,200	
	#4 (3 doses)	nd	nd	819,200	
	#5 (4 doses)	872(<10-4)	28,120	819,200	survived

*The p value, shown in parentheses, was derived from Z tests in which values for sera collected after vaccination were compared to the serum #1 preimmune value.
**Titer determined by using an antigen-capture Elisa [17].

AVA-IMMUNIZED MICE

The association between protection, serum GIA, and serum anti-PA titer was examined in mice immunized with the licensed human AVA vaccine. The decreased GIA of the prechallenge sera compared to the preimmune sera was significant for both the A/J and C57Bl6 mice (Fig. 7A). The sera were assessed for their spore GIA and for their anti-PA Ab Elisa titer. The results again suggested that that the serum GIA correlated with the prechallenge anti-PA titers of sera from immunized mice, as illustrated in Fig. 7B, but there were too few nonsurvivors to statistically determine the correlation between GIA, anti-PA titer, and protection.

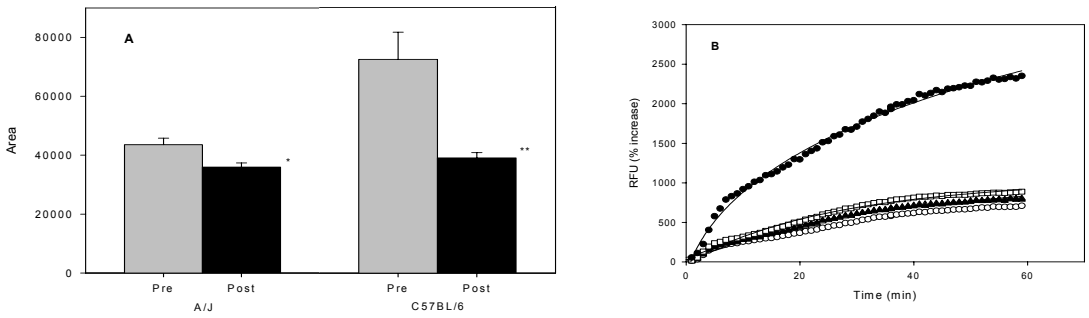

Figure 7. (a) Area under the fluorescence curve (RFU) of spores exposed to germinant after pretreatment with either preimmune serum (pre) or the prechallenge serum from the same animal post immunization with 3 – 4 doses of AVA (post). (b) Anti-PA titers and GIA of sequential sera from a C57Bl/6 mouse immunized with AVA. Serum #1 (preimmune): ●, serum #2: ❍, serum #3: △, serum #4: ▲, serum #5: ■

Shown in Figure 7 are the combined data from three experiments each of 3 A/J and 2 C57Bl6. The decreased GIA (mean areas) of the prechallenge sera compared to the preimmune sera was significant for both the A/J (*p = 0.023) and C57Bl6 (**p = 0.024) mice.

Table 2.

Mouse	Serum no. (No. vaccine doses)	Germination assay a coefficient*	Area	Anti-PA antibody titer**	TTD (day)
C57Bl/6	#1: preimmune	7,052	92,893	neg	
45(3)	#2: 1 dose	1,554 (0.02)	27,441	819,200	
	#3: 2 doses	1,376 (0.02)	31,831	819,200	
	#4: 3 doses	1,106 (0.01)	27,371	819,200	
	#5: 4 doses	1,150 (0.01)	36,055	819,200	survived

*The p value, shown in parentheses, was derived from Z tests in which values for sera collected after vaccination were compared to the serum #1 preimmune value.
**Titer determined by using an antigen-capture Elisa [17].

GUINEA PIGS

The association between serum germination inhibition activity and survival after challenge was examined in guinea pigs immunized with purified PA. Differences in germination of serum-pretreated Ames, as determined by comparing the Areas under the germination curve for spores pretreated with sera from the survivors and the nonsurvivors, indicated that the GIA of sera from survivors was significantly greater than that of sera from the nonsurvivors.

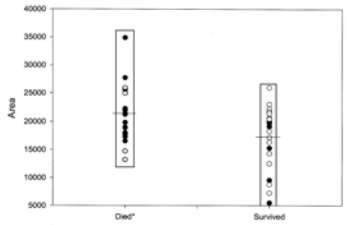

Figure 8. Prechallenge sera from the immunized guinea pigs was assayed for its GIA.

The sera were from guinea pigs immunized with recombinant PA (closed circles) or native PA purified from the Sterne strain (open circles) and then challenged im with Ames spores as described in the Materials and Methods. Differences in germination of serum-pretreated Ames were determined by comparing the Areas under the germination curve for spores pretreated with sera from the survivors and the nonsurvivors. *The mean area value of the animals that died, 20,710 [SEM 1118] was significantly greater than that of the animals that survived, 17,681 [SEM 1125], p = 0.04.

MONKEYS

The correlation between serum GIA, anti-PA titer, and survival in nonhuman primates immunized twice with dilutions of AVA and challenged by the aerosol route with Ames spores was studied. The increased GIA of sera from the survivors compared to that of the nonsurvivors was significant and correlated with protection (Table 3).

Table 3. Correlation between postchallenge survival of immunized monkeys and serum germination inhibitory activity

Animal	Vaccine group[a]	Survival[b]	Quantitative anti-PA IgG (ug/ml)	Fluorescence of germinant-exposed spores: Ratio of preimmune to immune-treated[c]
H312	1	S	84	2.2 (0.1)
L478	1	S	83	1.41(0.07)
89B115	1	S	730	1.83 (0.10)
16532	2	S	48	2.32 (0.37)
89B103	3	S	61	1.19 (0.21)
L651	4	S	BLQ[d]	1.02 (0.07)
Mean				**1.64** (0.12)[e]
L625	1	D	36	0.82 (0.04)
G6C	2	D	86	1.56 (0.13)
M049	2	D	55	2.34 (0.12)
H047	3	D	BLQ[d]	0.83 (0.03)
CH927	4	D	BLQ[d]	1.34 (0.07)
L921	5/placebo	D	BLQ[d]	0.94 (0.02)
L606	5/placebo	D	BLQ[d]	0.93 (0.11)
Mean				**1.29** (0.11)[e]

[a]Monkeys were vaccinated at t0 and 4 weeks later with the following dilutions of AVA: 1/12.5 (group 1), 1/25 (group 2), 1/50 (group 3), 1/100 (group 4), or a placebo of Alhydrogel in saline).

[b]Survival (S) or death (D) after challenge six weeks after the second vaccine dose by the aerosol route with 100 LD_{50} doses of *B. anthracis* strain Ames.

[c]Ratio of RFU of spores pretreated with the preimmune serum compared to the immune serum from the same animal.

[d]BLQ - Below the limit of quantitation, approx. 1 ug/ml.

[e]The mean ratio of the survivors was significantly greater than that of the nonsurvivors, p = 0.04. The mean fluorescence ratio correlated with the anti-PA titer (p = 0.007), but the titers did not correlate with survival.

CONCLUSIONS

A quantitative and sensitive assay for germination was developed using a microtiter kinetic reader for fluorescence spectrophotometry (Biotek FL600). The assay detected germination based on the increase in fluorescence of spores with time during their incubation in germination medium containing the fluorescent dye Syto-9. Defined media containing L-alanine, adenosine, and casamino acids, or L-alanine and L-inosine, stimulated germination with low levels of background fluorescence. Significant differences in germination kinetics were detected between spores pretreated with specific anti-PA IgG or nonimmune IgG. The germination inhibitory activity of anti-rPA Abs varied with the strain of *B. anthracis* used. The activity was shown using spores of the Ames and Sterne strains but not the Vollum1B strain. Using sera from mice, nonhuman primates, and guinea pigs vaccinated with PA, a potential correlation between protective immunity and germination inhibitory activity was shown. Purified rabbit polyclonal antibody prepared against whole spores also inhibited germination. Antigens specific to ungerminated spores that stimulate potentially protective antibodies are being identified, and their role in inhibition of germination and in immune protection will be determined.

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